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PURIFICATION OF N-ACETYLGALACTOSAMINIDASE BY ISOELECTRIC FOCUSI--ETC(U)
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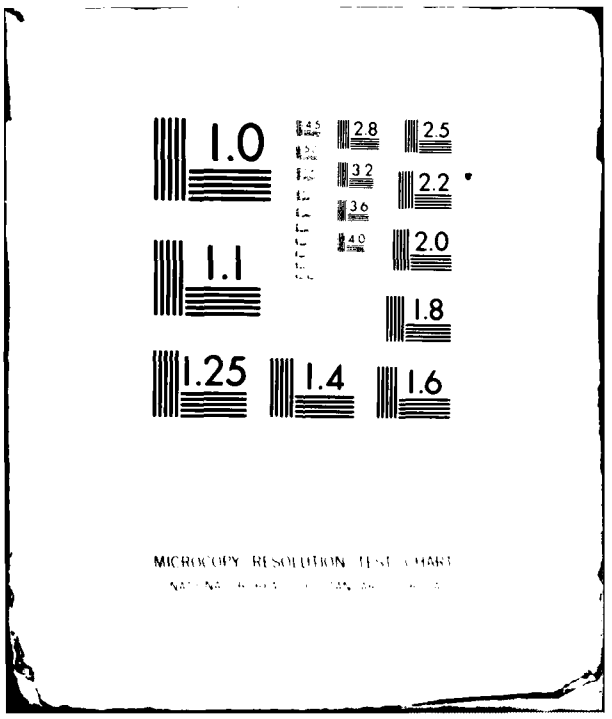
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Task No. NR 207-143

Annual Report No. 2

September 20, 1980

PURIFICATION OF N-ACETYLGALACTOSAMINIDASE BY ISOELECTRIC FOCUSING

by

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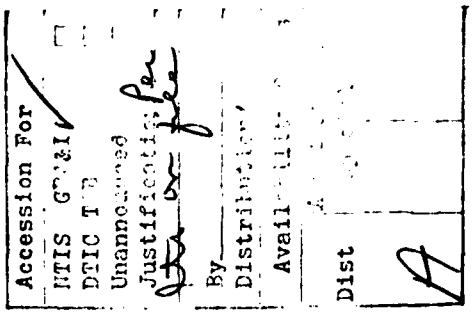


This is the second annual report of Contract N00014-78-C-0767, NR 207-143 which is directed at the development of enzymic methods for converting type A and B red blood cells to type O cells. The major thrust of our work in this period has been to develop a human source of this enzyme. Previous work has employed enzymes from bacteria. Even though the erythrocytes are treated in vitro and the enzyme is removed, it is difficult if not impossible to rule out the presence of small amounts of bacterial protein contaminating the erythrocytes. These might give rise to serious immunologic reactions such as anaphalaxis. Using enzymes from humans avoids this problem. Placentas were chosen as a source of this enzyme because of their availability and because they have long been used as a source of plasma for fractionation for human use.

The enzyme removes the terminal α-N-acetylgalactosamine from A substance to form type O and also removes the terminal α galactose from B substance to form type O. The present work has dealt with the characterization of the enzyme and its purification. In collaborative work with Dr. David Aminoff of the University of Michigan the partially purified enzyme has been shown to convert both A and B substances to O.

Purification

Placental Azyme has been purified 820 fold. The following steps were employed: ammonium sulfate fraction, isoelectric precipitation of impurities, precipitation of the enzyme with ethanol, and chromatography on DEAE cellulose. The yield was 19%. In all, almost 100 placentas were fractionated although much of the material awaits ethanol fractionation and chromatography. The studies listed delineate some of the properties of the enzyme which form the basis for the purification. These include demonstration that there is only one form of the enzyme by isoelectric focusing, that it is soluble and stable to freezing, the fractionation procedure is given in detail in a later portion of this report.

Isoelectric focusing was done on placental extracts to determine if a single enzyme existed or if there were multiple forms. Only a single activity was found. If multiple forms of the enzyme had occurred, it would have complicated the fractionation procedure since each form would have to be purified to determine which was the most useful. The crude homogenate was focused in a 5.4% T polyacrylamide gel which had been cross linked with 3% N, N' - methylene-bis-acrylamide. PEHA E, an ampholyte synthesized in our laboratory, was used at 2% concentration. The gels were polymerized by N,N,N',N' - tetramethylethylenediamine in 11 cm tubes. Samples containing 20-50 μg of protein in 5% sucrose were layered under a gel overlay containing 1% ampholyte and 2.5% sucrose.

The focused gels were either sliced into 2mm slices for assay, assayed as whole gels, or stained in Coomassie Brilliant Blue stain. The sliced gels underwent the following procedure. Two mm slices were each placed in 0.5ml of 25mM KCl (boiled for five minutes). The tubes were allowed to incubate for one hour at room temperature. The pH gradient was then determined by pH measurements on the diffusate of each consecutive gel slice and 0.3ml of 1M sodium acetate buffer, pH 4.0, was added to each tube. The slices were soaked overnight at 0°C and the diffusate assayed for Azyme activity.

Whole gels were assayed in a 20ml system containing the same relative amounts of buffer, substrate and water as a normal assay system. The reaction was terminated with 12.32ml of 15% Na_2Co_3. Gels for staining were soaked in a Coomassie Brilliant Blue stain containing 0.04% Coomassie Blue G-250 (65%) and 3.5% perchloric acid for two hours at room temperature, then destained in 7.5% acetic acid.

Protein was determined by the method of Bradford (1976). Only a single sharp peak of azyme activity was found which focused at 4.61 $\pm$ .07 (Fig. 1).

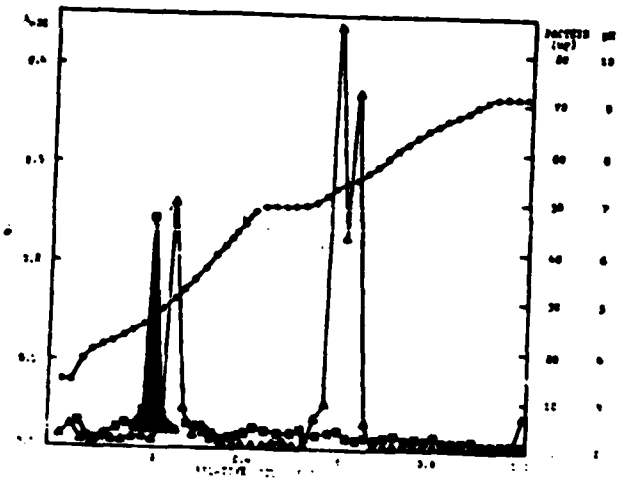
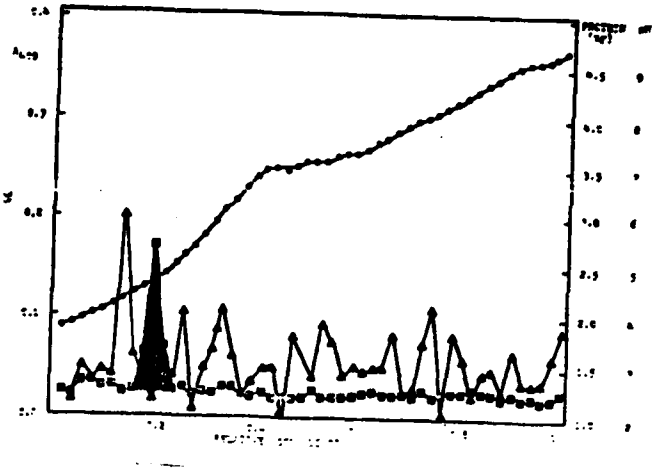


Fig. 1. Isoelectric focusing of B crude placental extract. A single well defined peak of Azyme activity (shaded area) is found.

The most highly purified material was also examined (Fig. 2).

Fig. 2. Isoelectric focusing of placental Azyme purified 820 fold. The Azyme (shaded area) has not been altered by the purification procedure. Note that the protein concentration scale is enlarged 15 fold over fig. 1.



The Azyme was still a single peak which focused at 4.68. By this criterion the Azyme had not been altered by the fractionation.

Effect of Freezing and Storage

A placenta obtained at the time of delivery was divided into 2 portions. One portion was homogenized and assayed. The other part was frozen at -18° overnight and then assayed. The frozen placenta had a slightly greater activity indicating that placental Azyme survives freezing and thawing. Long term storage of placentas did result in loss of activity. A placenta stored for 5 months at 18°C lost about 50% of its activity. The protein was also somewhat less extractable (15%) so that the homogenate made after 5 months of storage had about 60% of its original specific activity. Long term storage of placentas should be avoided.

Effect of Perfusion

The placental extract contains large amounts of hemoglobin and we explored the possibility that perfusion with saline might be a way of removing the red cells. The attempt was unsuccessful. Perfusion required several hours by which time 82% of the activity had been lost. Dr. Bent Boving, Professor of Anatomy and Gynecology/Obstetrics here, felt that any perfusion would have to be done within 15 minutes of delivery and should employ warm saline. This does not appear feasible and perfusion was abandoned.

Use of Proteinase Inhibitors

Several investigators have used inhibitors of proteolytic enzymes to help stabilize the enzyme. The effect of phenylmethylsulfonyl fluoride was examined. It is a general inhibitor of the group of proteolytic enzymes which use serine as part of their

catalytic site. Homogenates prepared in the absence of inhibitor have 82% as much activity as controls. Phenylmethylsulfonyl fluoride (0.5mM) was used in all subsequent homogenizations.

Ammonium Sulfate Fractionation

A large number of concentrations of ammonium sulfate, both neutral and acid, at room temperature and at +4° were examined to determine the optimal conditions for fractionation. The pH and temperature were not critical variables. The fraction which precipitated between 36% and 62% ammonium sulfate saturation at +4° was purified 4.1 fold in a yield of 94% and was used for further studies.

Precipitation of Impurities

When the ammonium sulfate was removed by dialysis a copious precipitate appeared. It was essentially devoid of activity. Precipitation conditions were optimized by examining the influence of pH on the precipitability of the impurities. A series of 8 pH's between 4.91 and 6.80 were examined. A pH of 5.4 was selected. Under large scale conditions it gave a yield of 76% for the step and a purification of 2.5 fold.

Ethanol Fractionation

A wide variety of conditions were explored for ethanol fractionation. These included ethanol concentration (5% increments from 0 to 50%), pH (4.4 and 5.4), temperature (-20°, 0° and room temperature), and ionic strength (0 and 0.02m acetate buffer). The activity remained in the supernatant. The purity was 5 fold and the yield 111%. These appear to be the optimal conditions for further work. While these studies were ongoing we wished simultaneously to undertake the purification by DEAE chromotography. For this purpose we fractionated at pH 5.4 in 50% ethanol at room temperature. After removal of the precipitate, the supernatant was placed at -18° for 2 hr after which it was recentrifuged. This precipitate was 5.7 fold purified but the yield was only 31%. It was devoid of β galactosidase or β-N-acetylglucosamidase activity under the conditions of assay. It is expected that initial precipitation of impurities at 0° followed by precipitation of Azyme at -18° will improve the yield of this step without loss of purity.

Chromatography on DEAE

The placental Azyme has been further purified by chromatography on DEAE cellulose. The ethanol precipitate was dialyzed, made 1.5% in sucrose and applied to a 1.5 x 30cm column of DEAE cellulose which had been equilibrated with 10 mM phosphate buffer at pH 6.5 at 4°. The enzyme was eluted by a 200 ml linear gradient which went from 0.0 to 0.5 m in NaCl. The gradient was started after 78 ml of buffer had passed through the tube. The Azyme appeared as a sharp peak at 178 ml, ie. 0.25 m NaCl (Fig. 3).

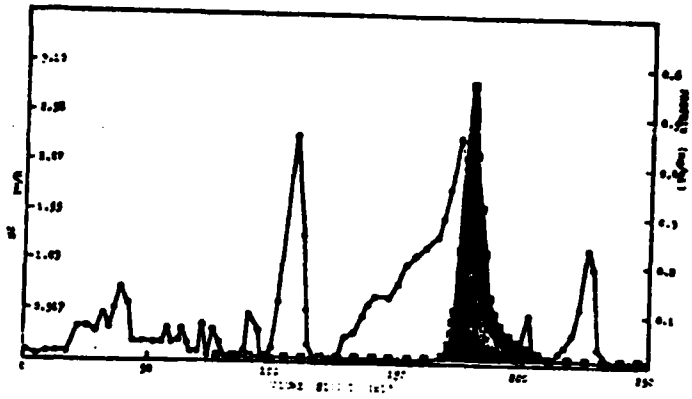


Fig. 3. Chromatography of placental Azyme (shaded area) on DEAE cellulose.

The purification procedure presently used for the early fractionation steps is described in the following paragraphs. Yields and purity for the preceding steps are summarized in Table 1.

Purification Procedure

Human placentas stored in a refrigerator from time of delivery were obtained daily from Hutzel Hospital. The membranes, cord and adhering clots were removed under running cold tap water and the placentas were cut into 2" squares and frozen at -16° in sealed plastic bags until use. Storage time varied from 4 to 41 days.

Phenylmethylsulfonyl fluoride (0.5 mM) was prepared by dissolving 1.57 g in 36 ml absolute ethanol at room temperature and adding the mixture to 8 liters of distilled water stirred on a magnetic stirrer at room temperature. After the phenylmethylsulfonyl fluoride was in solution it was moved to the cold room and used only after it had cooled. Two ml of solution were used per gram of placenta. Homogenization was done for 10 min at slow speed in a large capacity Waring blender. The homogenization was done in the cold room (+4 to +6°C) as were all subsequent steps unless mentioned. Usually 2 placentas were homogenized at a time. Each lot was made up of 8 or 9 placentas. In all 12 lots have been fractionated.

The homogenate was strained through 4 thicknesses of cheese cloth suspended in a 30 cm plastic funnel. Three funnels were required. The first 300 ml of filtrate was returned for refiltering since it often contained placental fragments. The process normally took about 40 hr including the time required for 2 half liter washes.

The filtrate was collected in a preweighed plastic bucket and the pooled filtrates weighed. Solid ammonium sulfate (100g/lb) was gradually added to the stirred filtrate and the stirring was continued for at least 2 hrs after additions. The suspension was allowed to stand for 16-20 hours and then centrifuged at room temperature through a Sharples desk top centrifuge at a flow rate of 2 l/hr or less. The precipitate (36% ammonium sulfate precipitate) was discarded and the supernatant brought to 62% saturation by adding

66.42g of ammonium sulfate to each pound of solution. After standing overnight, the suspension was centrifuged as previously and the precipitate was removed from the bowel and weighed. Typical yields were 0.17g ppt/g placenta. The precipitate was suspended in twice its weight of water and after stirring for about 1 hour was reprecipitated at 62% ammonium sulfate saturation by adding 0.406g ammonium sulfate/g water. After stirring for an additional hour the precipitate was removed by centrifuging for 1 hour in the Sovall SC2B centrifuge using the G3A rotor at 5000 rpm (4080 G) for 1 hr. The precipitate was suspended in its weight of water and dialyzed against several changes of water over a 60 hour period. The resultant suspension was titrated to pH 5.4 with 0.1n acetic acid and centrifuged in the Sorvall as previously and washed with one volume of water. The supernatants were stored frozen at -16° until further fractionation.

pH Optimum

The activity of the crude placental Azyme was assayed at 10 pH values between 2.31 and 11.13 at 37°C using p-nitrophenyl-2-acetamido-2-deoxy-α-D-galactopyranoside as substrate (Fig. 4).

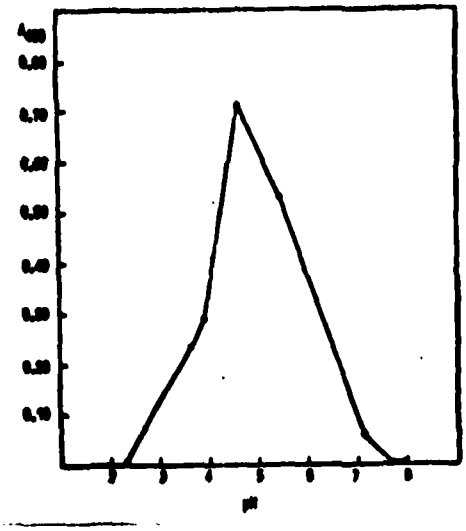


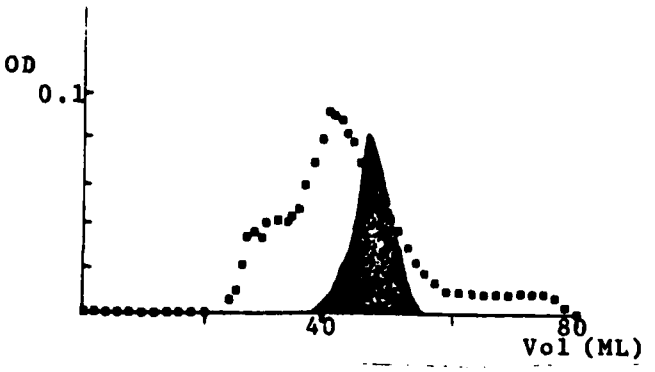
Fig. 4. pH optimum of placental Azyme.

The activity was best at pH 4.6 but 76% of the activity remained at pH 5.5 and 8% was left at pH 7.15. These findings form the basis for one important area of future work, finding optimal conditions for treating red cells.

Gel Filtration

Placental homogenate was also subjected to gel filtration on a 0.9 x 60 cm column of Sephacryl S 300 (Fig. 5)

Fig. 5. Gel filtration of placental Azyme (shaded area) on Sephacryl S 300.



The enzyme appeared to be single species with a molecular weight of 100,000.

Effect of Temperature on Enzyme Activity

Beutler and Kuhl (J. Biol. Chem. 247, 7195-7200 (1972)) reported that placental Azyme has an unusual temperature dependence. They found that the optimal activity was at 25° using 4 methylumbelliferyl-α-D-galactopyranoside as substrate and that it retained 40% of its activity at 4°. If this were so it would allow one to convert red blood cells at a low temperature. Our preparation of Azyme did not have this property when assayed with p-nitrophenyl-2-acetamido-2-deoxy-α-D-galactopyranoside. At 0° it had 9% as much activity as at 37°.

The assays were done at pH 4.0 in 0.1 m acetate buffer using Azyme which has been purified 58 fold. Each assay was incubated at the indicated temperature for one hour. Four different concentrations of substrate were used and the maximum velocity determined by a Lineweaver-Burk plot.

Temperature	37°	30°	22°	11.5°	0°
Relative velocity	1.0	.65	.53	.33	.09

The reason for the difference between our data and that of Beutler & Kuhl is unknown. Assays were done at different pH's, with different substrates, and with enzymes of different purity.

Heat Stability

The effect of heating on placental Azyme was examined since others have found that similar enzymes are quite stable. This property should be useful for heat inactivation of contaminating enzymes or heat labile viruses. Heating was also studied because some protein impurities might become insolubilized by this treatment. Azyme was quite stable to heat. Its activity was retained or increased during a 2-hour incubation at about 50°C. A heat labile inhibitor of Azyme was demonstrated. The heat treatment should increase the purity of the Azyme 2.2 fold.

Stability studies were done under the following conditions: 50°C in 0.063 M phosphate buffer at pH 6.5, 51°C in 0.10 M acetate buffer at pH 5.4, 51.5°C in 0.10 M acetate buffer pH 4.4 and 60°C in 0.10 M phosphate buffer pH 6.5. All samples which were heated at 50° retained their activity over a 2 hour period. At pH 6.5 the activity at 2 hours was 23% greater than the starting material, at pH 5.4 24% greater and at pH 4.4 1% greater. While there was little precipitate in the samples ran at pH 6.5 or 4.4, about 28% of the protein was insolubilized in the run at pH 5.4. The enzyme activity remained in the supernatant. Time studies indicate that in all 3 cases the heated mixture goes through a state where its activity is greater than that of the starting material. At pH 6.5 the maximal increase was 23% at 2 hours. At pH 5.4 it was 59% at 40 min and at pH 4.4 it was 15% at 10 min. The increase (Fig. 6)

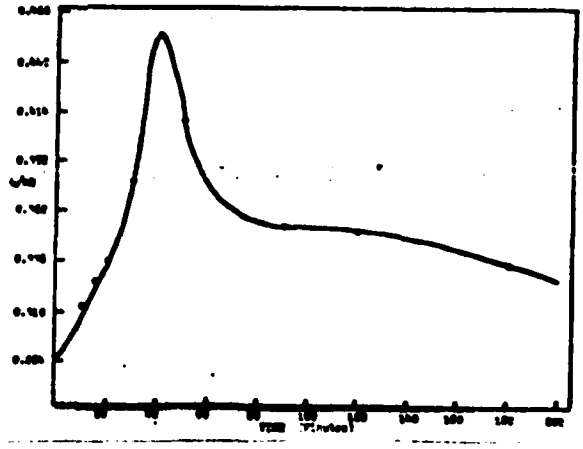


Fig. 6. Heat stability of placental Azyme at 50° C.

in activity suggested that a thermolabile inhibitor was present. This was further demonstrated by performing assays of mixtures of crude and purified Azyme. The activity of the mixture was less than that of the two fractions measured separately. The purest fraction studied gave a result 76% lower than expected. Heating the enzyme mixtures for 40 min at 50° removed much of the inhibition suggesting that the inhibitor was thermolabile. A stability was also done at 60°. The material was rather labile under these

conditions. After 10 minutes 50% of the activity had been destroyed. The end product of the reaction, N-acetylgalactosamine, did not protect the Azyme from inactivation under these conditions. The N-acetylgalactosamine was 14.6 mM. Its K_I is 4.6 mM. The stability to urea and guanidine was also examined. Ninety % of the activity remained after 1/2 hour at room temperature in 4M urea but only 33% was left under these conditions when 8 M urea was used. The urea denatured enzyme did not reactivate when the urea was diluted. Azyme incubated at 50° for 30 min in 1 M urea had 79% of its original activity, in 2 M urea 43% was left and in 4 M urea only 5%. Guanidine hydrochoride also denatured the enzyme. After 30 minutes at room temperature 85% was left in 1 M reagent, 37% in 2 M and 2% in 4 M.

Comparison With Azyme From Liver

Dean and Sweeley (J. Biol. Chem. Chem. 254 10001-10005 (1979)) have purified α-N-acetylgalactosaminidase from human liver. We wished to compare its specific activity to that of placental enzyme to determine which is the better source.

This comparison was done by assaying our placental extracts by the procedure used by Dean and Sweeley. Their assay gives 7 times as much product as ours primarily because our assay uses only a small amount of substrate which is quite expensive. Under our assay conditions the substrate concentration is 0.36 mM. Dean and Sweeley use 5.0 mM substrate. The K_m at 37° is 7.69 mM. Comparison of the ratio of activity of placenta by their assay per mg of protein to their value for liver indicates that placenta is 6.4 times richer. It is of course much more available.

Publication

pH gradient flattening in isoelectric focusing in long polyacrylamide gels. Ray Rapaport, Andrew Jackiw and Ray K. Brown, Electrophoresis 1 In press (1980).

Table 1: Purification of Azyme from Human Placenta.

Step	Total Protein (g)	Enzyme Units (umol hr^{-1})	Specific Activity (umol hr^{-1} mg^{-1})	Yield (%)	Purification (-fold)
1. homogenate supernatant	153	1963.6	0.0128	100	1.00
2. 36% ammonium sulfate	134	2155.4	0.0161	110	1.26
3. 36%-62% ammonium sulfate	35.5	1845.5	0.0520	94.0	4.06
4. isoelectric precipitation	10.7	1408.7	0.131	71.7	10.23
5. 50% ethanol fractionation	0.110	439.8	0.744	22.4	58.12
6. DEAE-cellulose column chromatography	0.00260	373.0	10.54	19.0	823.40

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An enzyme which converts type A and type B substances to type O is being purified from human placenta as a reagent for preparing type O, universal donor, red blood cells. The enzyme has been purified 820 fold using ammonium sulfate fractionation, isoelectric precipitation of impurities, ethanol fractionation and chromatography. The crude enzyme is in part complexed to a thermolabile inhibitor. The enzyme is unusually stable to heat and has a pH optimum near pH4.6 using p-nitrophenyl-2-acetamido-2 deoxy-α-D-galactopyranoside as substrate. Collaborative studies with Dr. David Aminoff of the University of Michigan have

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20. Abstract (Continued)

shown that the enzyme removed the terminal N-acetylgalactosamine from A substance and the terminal galactose from B substance to form O substance.

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